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Short Communication

Evaluation of fungal-specific fluorescent labeled echinocandin probes as diagnostic adjuncts

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The diagnosis of invasive fungal infections from radiographic imaging is non-specific and problematic. As a first step toward increasing specificity, we describe the development of a broad-spectrum fungal-specific targeting molecule, which when modified with a fluorescent label fully retains its targeting properties, and provides a basis for future imaging applications.

Keywords diagnostics, invasive fungal infections, fluorescence labeled anti-fungal, caspofungin

Introduction

Invasive fungal infections (IFI) are a growing threat to human health due to immunocompromising diseases [1]. In most situations where IFI diagnosis is considered, the clinical presentation is often non-specific and can be caused by a wide range of infectious organisms, underlying illness, or complications of treatment. Successful IFI diagnosis is further complicated due to uncertainties and controversies in disease definition and in selecting standardized methods for establishing the diagnosis [1,2]. Fungal cell wall components such as glucans and galactomannans, which are actively shed during growth and development, are the basis for biomarker-based commercial antigen assays for rapid diagnostic testing [3], but their value is limited by the potential for false-positive and false-negative results due to an assortment of factors [4–8]. In recent years, standardization of protocols for PCR amplification of organisms, such as *Aspergillus* spp. from blood and respiratory samples [9] has aided in the diagnosis of these infections, especially since cultures are often negative [10].

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Imaging is an important part of the diagnosis of diseases, such as invasive aspergillosis (IA). Characteristic images from conventional X-rays and more advanced computed tomography (CT) can be used to identify disease lesions in neutropenic patients and help manage IA. Importantly, the CT halo sign is a transient finding that provides a probable diagnosis of early invasive pulmonary aspergillosis. Patients with a halo sign at baseline are more likely to have a satisfactory treatment response than those without this indicator [11]. Unfortunately, diagnostic imaging is inherently non-specific and must be used in combination with other clinical and microbiological factors. An important adjunct for imaging would be the development of fungal-specific probes that can be used to specifically image infecting pathogens in the lungs of patients. In this report, we describe the development of novel fungal-specific probes that have the potential to be used for diagnostic imaging.

Our approach takes advantage of highly specific and sensitive antifungal drugs with known safety properties as targeting molecules. By coupling a fluorophore that can be visualized, the new probe would be able to detect the presence of fungal pathogens. The echinocandin drug caspofungin (CSF) binds with high affinity to its fungal target glucan synthase [12,13], which is not found in humans. This fungal specificity potentially makes it a powerful and highly discriminating diagnostic tool for the sensitive detection and visualization of fungal pathogens, when

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coupled with a suitable label [4]. The antifungal molecule caspofungin (CSF) shows broad activity against clinically important *Candida* and *Aspergillus* spp. [12]. It contains two aliphatic primary amino groups that are convenient sites for chemical modification, which facilitate the engineering of broad-spectrum labeled probes.

Methods and materials

Synthesis of BODIPY-labeled drugs

A derivative of CSF using BODIPY (BOD) (Fig. 1) was produced by incubating BODIPY-succinimidate with pure CSF in the presence of triethylamine as a proton acceptor in DMF. The crude product was purified by TLC silica gel chromatography and characterized using mass spectroscopy, fluorescence and UV spectroscopy (not shown). It was critical that the modified agent retained its specificity and sensitivity to the fungal target. To test these properties, the antifungal activity of the modified and unmodified root compounds were evaluated and found to be effectively unaltered by the presence of label (*C. albicans* $MIC_{unlabeled} = 0.06$ μg/ml v. $MIC_{labeled} = 0.12$ μg/ml) confirming that it retained its inherent potency. BODIPY coupled to posaconazole (POS) was formed by modifying a single hydroxyl group of posaconazole with succinic anhydride (Fig. 2).

Incubation of the acylation product with 4-nitrophenol in the presence of N,N′-Dicyclohexylcarbodiimide (DCC) yielded a new compound consistent with the formation of an activated ester (compound **II**). Incubation of this compound with ethylenediamine resulted in a characteristic absorption at 405 nm of nitrophenolate anion, which was indicative for acylation of the diamine by posaconazole activated ester. Incubation of the ester with aminobutane derivative of BODIPY fluorophore (compound **III**) yielded fluorescent posaconazole-BODIPY adduct **IV**, with expected light absorption spectrum.

Cell labeling

To illustrate the potential of CSF-BOD and POS-BOD for visualizing fungal cells, the reagent was used to probe for the presence of *Candida* and *Aspergillus* species in a variety of matrices including solid and liquid growth media. The clinical *A. fumigatus* wild type strain R21 and *Candida albicans* ATCC strain 90028 were used for all the experiments. For *Aspergillus*, one drop of yeast extract peptone dextrose (YPD) agar was placed in the upper right corner of each well of a 15-well multi-test slide followed by the addition of 10 μl of saline containing 10^5 conidia of R21. The slide was placed in a sterile Petri dish with distilled water to provide a moist environment and incubated in a 37°C incubator for 10–16 h to facilitate germination and growth of hyphal elements. A 10 μl aliquot of CSF-BOD (170 ng/ml) or POS-BOD (150 ng/ml)was added to each well and incubated for 6 h at 37°C, followed by washing three times with sterile water and drying by vacuum. For *Candida*, an overnight culture of *C. albicans* was grown, washed by centrifugation and resuspended in dH_2O. The yeast cells were added to RPMI and incubated at 200 rpm for 1 h at 37°C to form germ tubes before being washed and resuspended in a 1 ml solution of CSF-BOD (120 ng/ml) or POS-BOD (150 ng/ml). The cells with drug were incubated again at 200 rpm for 1 h at 37°C, washed and resuspended in 50 μl of dH_2O. A 15-well slide was prepared using a poly-L-lysine to tightly adhere cells to the wells. A 10 μl aliquot of *Candida* cells was placed on each well and incubated for 10 min, aspirated off, and 1 μl Slow Fade Antifade reagent was added to prolong the fluorescent

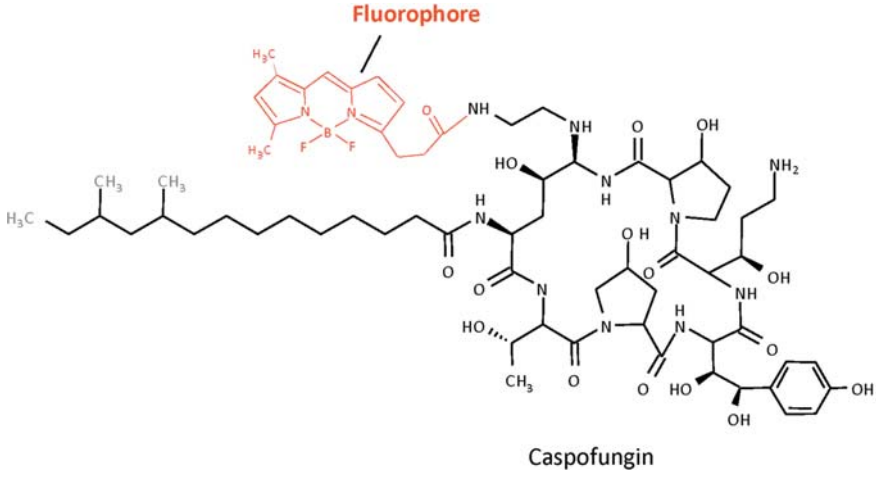

Fig. 1 Chemical structure for CSF-BODIPY.

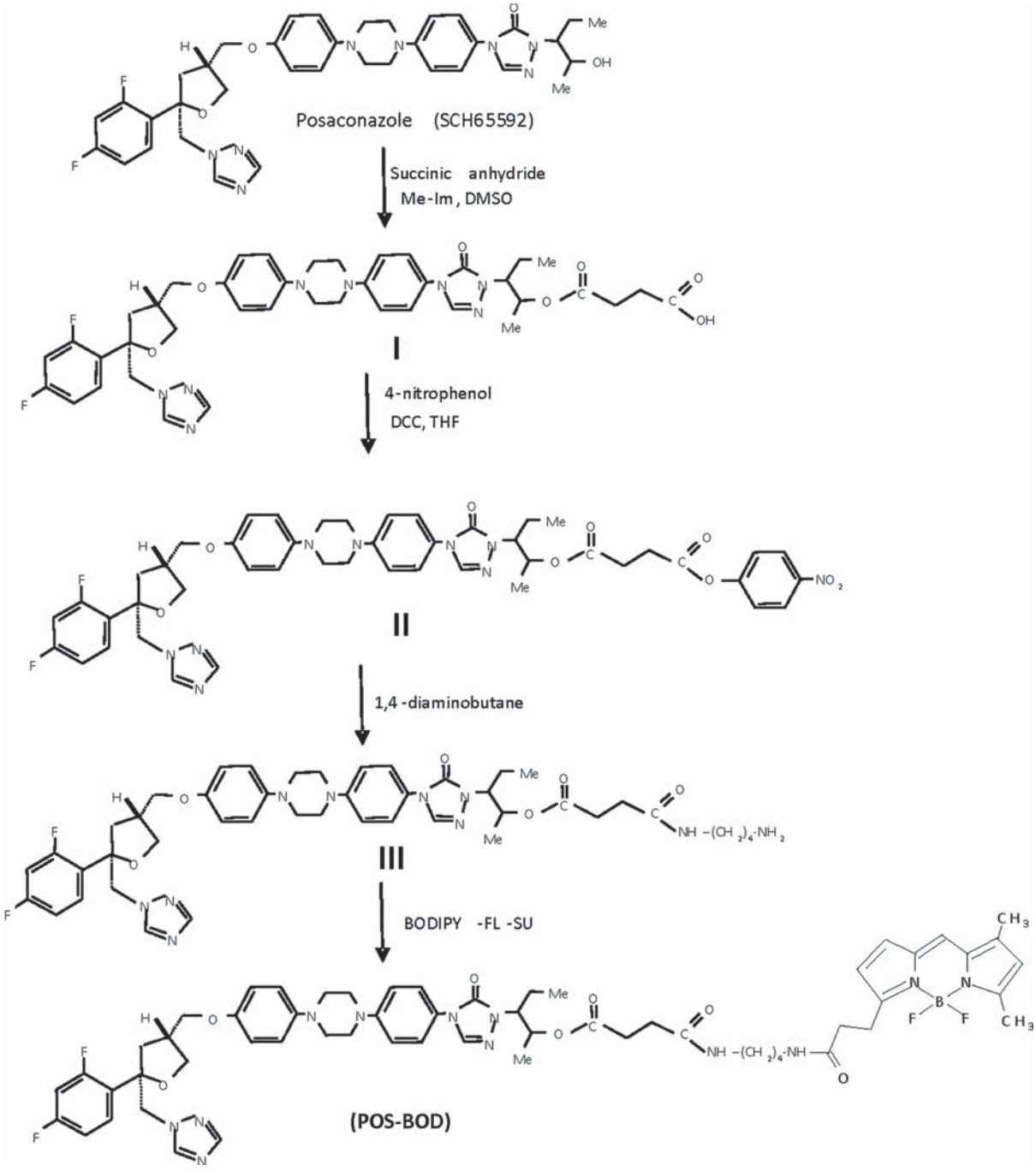

Fig. 2 Chemical synthesis steps required for chemical adjunct, POS-BOD.

life and moisture level of the cells on the each slide. Each slide was observed under 100× magnification with the total internal reflection objective lens (TIRF) of a Nikon Eclipse 90i fluorescent microscope. All 15 wells of the slides were examined individually using *Volocity*® 3D Image Analysis Software (PerkinElmer). Individual cells, hyphal elements and clusters of cells were visualized, analyzed, and captured in bright field lighting and repeated under a Green Fluorescent Protein (GFP) light setting.

Results

Incubation of *C. albicans* at the MIC (120 ng/ml) for 1 h at 37°C resulted in generalized fluorescence in the mother

cell membrane with slightly more defined punctate fluorescence along the germ tube axis toward the growing tip, consistent with the putative intracellular vesicle trafficking of glucan synthase from clustered golgi vesicle complexes (Fig. 3A). Under 6 h and 37°C conditions, *A. fumigatus* showed bright fluorescence in the spore but more diffuse labeling of the surface of the hyphal elements toward the growing apex (Fig. 3B) consistent with a membrane location for glucan synthase.

As expected, labeling was highly temperature sensitive with maximal labeling observed over the 6 h period at 37°C. Caspofungin and BODIPY alone failed to produce any labeling (results not shown). The level of binding was greatly reduced in a well-characterized *fks*1-S645F mutant [14], which has diminished sensitivity of glucan synthase for echinocandins consistent with the probe binding to its intended target (Fig. 3C).

To ensure the specificity of this method for fungi, representative Gram negative and Gram positive bacteria: *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Serratia marcescens*, *Staphylococcus aureus* and *Escherichia coli* were grown and labeled under the same conditions. No fluorescence was observed in any cells (results not shown).

To further confirm the targeting properties of CSF-BOD, posaconazole, a potent and selective inhibitor of the sterol biosynthetic enzyme cytochrome p 450 lanosterol 14α demethylase, was modified with BODIPY (POS-BOD). Under the same labeling conditionsas CSF-BOD, POS-BOD showed generalized fluorescence labeling of mother cell and elongating hyphal elements with both *Candida* and *Aspergillus*. Pretreatment with unlabeled posaconazole or voriconazole greatly diminished or eliminated the fluorescence signal, while pretreatment with caspofungin had

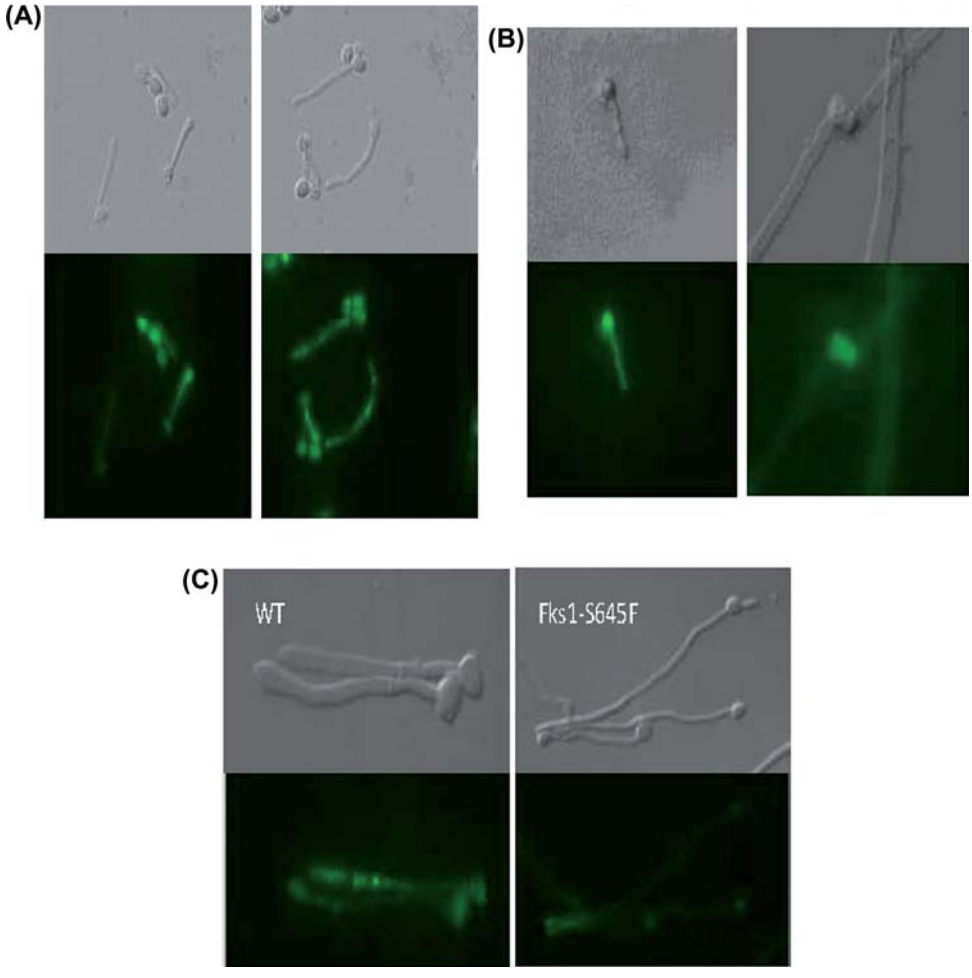

Fig. 3 Generalized labeling of *Candida albicans* and *Aspergillus fumigatus* with CSF-BODIPY. (A) *C. albicans* was labeled for 1 h with CSF-BODIPY at 0.12 mg/ml and (B) *A. fumigatus* was labeled for 6 h at 0.17 mg/ml. Figures show light microscopic image (100ü) and corresponding fluorescence image captured on Nikon 90i fluorescence microscope. (C) Fluorescent labeling of *C. albicans* hyphal germ tube from wild type and echinocandin-resistant *fks1*-S645F strain with CSF-BODIPY. Wild type and mutant strains were labeled for 2 h with 0.12 mg/ml.

little effect on labeling of cells by POS-BOD, as expected since they bind to separate targets (results not shown).

Pretreatment of cells (*C. albicans* 1 h and *A. fumigatus* 2 h) at one dilution below the MIC with four different azoles, voriconazole (Pfizer), itraconazole (Janssen), posaconazole (Merck) and fluconazole (Pfizer), followed by CSF-BOD labeling described above had no effect on labeling. All samples showed the same fluorescence intensity with and without pretreatment consistent with azoles binding to a separate intracellular target. However, cells pretreated with the echinocandins, anidulafungin and micafungin, prior to standard labeling with the CSF-BOD probe eliminated the labeling. Pretreatment with caspofungin intensified the fluorescence most likely due to an efficient exchange-binding phenomenon of modified drug for unlabeled bound drug (not shown). The data is consistent with the echinocandin drugs competing tightly with the probe for glucan synthase [13,15].

Discussion and conclusion

In this study, CSF-BOD was able to detect various fungal structures including germinated cells and hyphal elements, and it specifically labeled fungal cells without cross-reacting with common pathogenic bacteria. The experimental labeling occurred at a compound level consistent with the high sensitivity and specificity of the root drug for its fungal target. Furthermore, we demonstrate the specificity of the CSF-BOD by evaluating labeling following competition with azole and echinocandin pre-treatment, and in the presence of a *fks* mutation.

Overall, this pilot study establishes a preliminary basis for fungal-specific targeting molecules that with the appropriate labels can be used as an adjunct to support the diagnosis of invasive fungal infections. Validation of the probes as viable clinical diagnostic tools will require *in vivo* efficacy studies and an assessment of serum and other host factors. Such an approach when coupled with existing high resolution imaging modalities using radiographically-visible labels has the potential to decrease morbidity and mortality in immunocompromised patients by facilitating more accurate diagnostics.

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